Binding kinetics of immobilized antibodies in a flow immunosensor

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Abstract

This study investigates the binding kinetics of immobilized antibody in a solid-phase displacement immunosensor conducted in flow under nonequilibrium conditions. The experimental system studied employs a monoclonal antibody, specific for cocaine and its metabolite, benzoylecgonine, immobilized onto agarose beads. After saturation of antibody binding sites with the fluorophore-labeled antigen, fluorescein–cadaverine–benzoylecgonine, the system is placed in a buffer flow. Injection of unlabeled antigen causes displacement of proportional amounts of antibody-bound labeled antigen, which is detected downstream. Using a repetitive displacement technique, we have characterized the kinetics by determining the displacement efficiency and apparent dissociation constant at various flow rates and different antibody surface densities. The experiments were performed with several antigen concentrations to evaluate the reliability of the repetitive displacement technique. The amount of displaced labeled antigen was found to be a function of both the density of antibody-bound labeled antigen and the amount of injected unlabeled antigen. Upon repetitive sample injections, an exponential decrease of displaced labeled antigen is observed, corresponding to a similar decrease in the undissociated fraction. Further kinetics analysis reveals that the displacement efficiency is dependent on the flow rate and the antibody surface density. The antibody immobilized at low density is much more sensitive to changes in the flow rate than the antibody immobilized at high density, as indicated by the decrease in the displacement efficiency and increase in the apparent dissociation constant (k_d) with the increase in flow rate.

Keywords: Flow immunosensors; Immobilized antibodies

1. Introduction

Immobilization of biomolecules has played a critical role in the utilization of immunoassay and biosensor technologies. Taking advantage of the specificity of antibody binding, solid-phase immunosensors have proven to be reliable and appropriate for a wide variety of applications, including clinical and environmental testing. To increase the performance capabilities of solid-phase immunosensors, it is important to examine the parameters that affect the kinetics of antibody binding at solid–liquid interfaces. In particular, understanding how immobilization affects the affinity of the antibody for the antigen is critical. While classical concepts have been applied to characterize the interactions of antigen with immobilized antibodies under equilibrium conditions [1–5], these systems do not adequately explain the nonequilibrium kinetics of antibody–antigen binding at solid–liquid interfaces [6]. A sensor that operates under such nonequilibrium conditions is the continuous-flow immunosensor [7]. In the flow immunosensor, a monoclonal antibody is immobilized onto a solid support, saturated with fluorophore-labeled antigen, and placed in a buffer flow. When unlabeled antigen is introduced, a proportional amount of fluorophore-labeled antigen is displaced from the binding sites of immobilized antibodies and subsequently detected downstream. The flow immunosensor is exceptionally attractive for fast analyses since it does not require an incubation period and provides a signal in less than a minute.

In previous studies, we investigated the effect of physical and chemical parameters on the kinetics of immobilized antibodies in the flow immunosensor, and
developed a preliminary theoretical framework utilizing mass law to describe the kinetics of antigen binding to immobilized antibodies in flow under nonequilibrium conditions [8,9]. In the present study, various concentrations of loaded antigen and different surface densities of antibody were used in a repetitive displacement assay in order to determine the displacement kinetics at changing flow rates. The antibody binding kinetics were characterized by determining the displacement efficiency and the apparent dissociation rate constant.

2. Experimental

2.1. Antibody immobilization

A monoclonal antibody (mouse IgGλ) specific for both cocaine and its major metabolite, benzoylecgonine, was obtained as ascites from Biodesign Corporation (Kennebunkport, ME). A 10 mg Membrane Affinity Separation System cartridge (Nygene Corp., Yonkers, NY) was used to isolate the IgG fraction from the ascites, as described previously [8]. Different densities of the IgG anti-benzoylecgonine antibody were immobilized on tetryl chloride-activated Sepharose 4B (Pharmacia, Piscataway, NY) using the protocol described previously [9]. The high- and low-antibody-density Sepharose had 4.5 and 0.9 pmole antibody immobilized on 1.0 mg of gel (approximately 4 ml of hydrated gel), respectively.

2.2. Repetitive displacement experiments

Antibody-coated Sepharose and a 100-fold molar excess of fluorophore-labeled antigen (a fluorescein–cadaverine–benzoylecgonine conjugate [8]) over immobilized antibody were incubated at 4 °C. For each experiment, a 50 mg aliquot of the Sepharose matrix was dispersed into a small, disposable column (Isolab, Inc., Akron, OH) and phosphate-buffered saline was pumped through to remove unbound fluorophore-labeled antigen.

The apparatus for measuring the antigen response in the flow immunosensor included a Jasco 821-FP fluorimeter (Jasco International Co., Tokyo, Japan), equipped with an 8 μl flow cell, a Hewlett-Packard Integrator, a Rabbit-Plus® peristaltic pump (Rainin Instruments, Emeryville, CA), and a Rheodyne five-way valve (Rainin Instruments, Emeryville, CA) employed as a low-pressure sample injector. The column eluent was monitored at an excitation of 490 nm and emission of 520 nm. When the background fluorescence was less than 0.04 arbitrary fluorescence units, 200 μl samples containing identical amounts of cocaine in buffer were injected repeatedly into the buffer flow and the fluorescence of the displaced labeled antigen was measured. The total amount of labeled antigen Ag* bound to the immobilized antibody, (bound Ag*)t=0, was determined by repeated injections of large amounts of cocaine samples (100-fold molar excess of cocaine to immobilized antibody) until the column was depleted of labeled antigen. For each density of immobilized antibody, the density of active antibody was determined by measuring the amount of displaceable labeled antigen. All calculations are relative to this value, which is independent of any denatured or inactive antibody.

3. Results

A characteristic depletion pattern of labeled antigen from the immobilized antibody was observed for each repetitive displacement experiment. Fig. 1 depicts the labeled antigen displaced from the matrices containing immobilized anti-cocaine antibody at two different densities of 50 and 245 pmole per 50 mg of matrix. The upper panel illustrates the displaced labeled antigen from columns containing the low-density antibody matrix detected after antigen injection at three different molar ratios of antigen to immobilized antibody (1, 10 and 100), whereas the lower panel depicts the data from the same experiment performed with columns containing the high-density antibody matrix. The samples were injected at flow rates of 0.5, 0.75 and 1.0 ml min⁻¹. The data illustrate that (i) as the molar ratio of total antigen injected was increased, a corresponding increase in displaced labeled antigen was observed; (ii) the increase of antibody surface density by a factor of five is associated with a four- to six-fold increase in the displaced labeled antigen; and (iii) the highest level of displaced labeled antigen was measured at the lowest flow rate.

Using equations derived previously [8], the undissociated fraction of labeled antigen was calculated from the difference between total bound labeled antigen and the amount displaced after each addition of unlabeled antigen as follows:

\[
\theta = \frac{(\text{bound } Ag^*)_{t=0} - \Sigma (\text{displaced } Ag^*)}{(\text{bound } Ag^*)_{t=0}}
\]

where Ag* represents the amount of labeled antigen. Fig. 2 illustrates the calculated time values for the undissociated fraction (θ) plotted as a function of the displacement time. The period available for displacement was determined by dividing the volume of the column containing the immobilized antibody by the flow rate. Different slopes were observed for the various flow rates and amounts of loaded unlabeled antigen, reflecting differences in the rate of depletion of the labeled antigen from the column. At each flow rate and amount of unlabeled antigen, the exponential decrease of the undissociated fraction occurred faster on the low-density matrix, suggesting an inverse relationship.
Fig. 1. Effect of flow rate on displaced labeled antigen at low (upper panel) and high (lower panel) antibody densities. Samples of cocaine were injected repeatedly at flow rates of 0.5, 0.75, and 1.0 ml min⁻¹ into 200 μl columns containing 50 pmole (upper panel) or 245 pmole (lower panel) of immobilized anti-cocaine antibody. The values found by each displacement of labeled antigen from the matrices are shown. The antigen was introduced at molar ratios of 1, 10 and 100 times that of immobilized antibody. The data represent the mean of two experiments.
Fig. 2. Effect of flow rate on displacement at low (upper panel) and high (lower panel) antibody densities. After each application of antigen, the undissociated fraction was plotted vs. the cumulative time of exposure of the column to antigen at flow rates of 0.5, 0.75, and 1.0 ml min$^{-1}$. The data represent the mean of two experiments.

between antibody density and the rate of labeled antigen depletion.

Next, the effect of flow rate on the displacement efficiency ($D_e$) was calculated for the data from an equimolar amount of loaded antigen, using the following relationship:
those that maintained a high density of labeled antigen on the column throughout the experiment and maximized the interaction time of the loaded antigen and the antibody (i.e., high density at 0.5 ml min⁻¹ flow rate). An increase in the flow rate from 0.5 to 1.0 ml min⁻¹ caused significant changes in the rate of labeled antigen released.

Finally, the effect of antibody density at different flow rates on the apparent dissociation constant \( k_d \) was analyzed using

\[
k_d = -\frac{\ln \theta}{t}
\]

where the time period available for displacement \( t \) was again determined by dividing the volume of the column containing the immobilized antibody by the flow rate. The term 'apparent' dissociation reflects the fact that the constant is calculated from the amount of labeled antigen released from the column. This constant is a function not only of the actual \( k_d \) of the antibody but also of other factors such as nonspecific binding and accessibility of the antigen-binding sites. The data demonstrate a reciprocal relationship between these two parameters and the density of the immobilized antibody (Fig. 4). An approximately five-fold increase in antibody density is associated with a two-fold decrease of the apparent dissociation constant. The apparent \( k_d \) values are dependent on both antibody density and flow rate. Furthermore, an increase of the flow rate from 0.5 to 1.0 ml min⁻¹ is associated with a two-fold increase in the apparent \( k_d \) values, independent of the immobilized antibody density.

**Fig. 3.** Relationship of antibody density, flow rate, and displacement efficiency. Antigen was injected repeatedly at flow rates of 0.5, 0.75, and 1.0 ml min⁻¹ into columns containing antibody at the high and the low antibody densities described in Fig. 1.

Using

\[
D_e = \frac{\text{(displaced Ag*)}}{\text{(loaded Ag)}} \frac{1}{\theta}
\]

where the 'loaded Ag' was constant during the repetitive displacement experiments and \( 1/\theta \) represents the factor that corrects for depletion of label with time. Fig. 3 shows the effect of antibody density on the displacement efficiency as a function of the injection number. For the low-density matrix, the calculated displacement efficiency of the first injection was higher than that of the high-density matrix. In an ideal system, \( D_e \) should be constant. However, the only conditions that produced a relatively consistent value were

**Fig. 4.** Apparent dissociation constant \( k_d \) as a function of flow rate for both low and high antibody densities. The values were calculated using Eq. (3), where time refers to the time available for dissociation at each flow rate.
4. Discussion

To increase the performance capabilities and improve the design of solid-phase immunosensors working in flow conditions, the understanding of factors influencing the antibody binding kinetics is pivotal. To this end, the role of flow rate and antibody density in a continuous-flow immunosensor has been investigated here. The data demonstrate that the flow rate has a major impact on the dissociation kinetics. At the higher flow rate, the displacement efficiency for the low-antibody-density assay is decreased, due to a reduced time period available for displacement. An increase of the flow rate from 0.5 to 1.0 ml min⁻¹ is associated with a two-fold increase in the apparent kₐ values, independent of the density of the immobilized antibodies.

Increasing the density of the immobilized antibody reduced the apparent dissociation rate. This observation may reflect a greater opportunity for dissociated labeled antigen to rebind to the higher-density antibody. If rebinding is occurring, one would predict that, as the flow rate increases, the rate of dissociation (the apparent kₐ) would increase as well. Such an increase is indeed observed. This dependence of kₛ on antibody density may only apply to nonequilibrium systems. Pellequer and Van Regenmortel [4] report that dissociation rates calculated for immobilized antibodies under equilibrium conditions are not sensitive to antibody density over a 100-fold range of concentration.

While flow rate and antibody density have been identified as critical parameters in our study, additional factors are also likely to improve the performance of immunosensors. Such factors include the surface properties of the solid support, the geometry and diameter of solid support pores, and the method used for the immobilization of the antibody. In this study, homogeneous monoclonal antibodies were immobilized onto porous matrices via their primary amino groups. Since an antibody molecule contains multiple primary amino groups, it is impossible to control the specific amino group(s) used for immobilization. Consequently, antigen binding is likely to be affected to a certain extent by immobilization. For example, an attachment site in close proximity to the antigen binding region might cause changes in affinity due to steric-hindrance effects. Factors such as loading, surface density, and orientation are known to affect the binding parameters of immunosorbents [10]. It remains to be determined, however, to what extent the binding kinetics could be influenced by site-directed immobilization techniques such as carbohydrate-directed antibody coupling procedures, which are known to minimize impairment of antibody binding functions.

In summary, this study examines the interdependence of several variables that must be considered in immunosensor development. For the flow immunosensor, a complex relationship was found to exist between surface antibody density, flow rate, displacement efficiency, and apparent dissociation constant. This understanding of the displacement kinetics at a solid–liquid interface under flow conditions allows for the modulation of sensor detection limits, lifetime, and sensitivity. While the evaluation of additional parameters, such as antibody affinity of the immobilized antibody and immobilization procedure, requires further experiments, our analytical approach allows for the design and efficient optimization of the continuous-flow immunosensor.

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References


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